

NASA TECHNICAL MEMORANDUM

NASA TM-78268

ISOELECTRIC FOCUSING OF RED BLOOD CELLS IN A
DENSITY GRADIENT STABILIZED COLUMN

By Adam J. K. Smolka and Teresa Y. Miller
Space Sciences Laboratory

February 1980

(NASA-TM-78268) ISOELECTRIC FOCUSING OF RED
BLOOD CELLS IN A DENSITY GRADIENT STABILIZED
COLUMN (NASA) 27 p HC A03/MF A01 CSCL 07D

N80-19217

Unclas
G3/25 47523

NASA

*George C. Marshall Space Flight Center
Marshall Space Flight Center, Alabama*



1. REPORT NO. NASA TM-78268	2. GOVERNMENT ACCESSION NO.	3. RECIPIENT'S CATALOG NO.	
4. TITLE AND SUBTITLE Isoelectric Focusing of Red Blood Cells in a Density Gradient Stabilized Column		5. REPORT DATE February 1980	
		6. PERFORMING ORGANIZATION CODE	
7. AUTHOR(S) Adam J. K. Smolka* and Teresa Y. Miller		8. PERFORMING ORGANIZATION REPORT #	
9. PERFORMING ORGANIZATION NAME AND ADDRESS George C. Marshall Space Flight Center Marshall Space Flight Center, Alabama 35812		10. WORK UNIT NO.	
		11. CONTRACT OR GRANT NO.	
12. SPONSORING AGENCY NAME AND ADDRESS National Aeronautics and Space Administration Washington, D.C. 20546		13. TYPE OF REPORT & PERIOD COVERED Technical Memorandum	
		14. SPONSORING AGENCY CODE	
15. SUPPLEMENTARY NOTES Prepared by Space Sciences Laboratory, Science and Engineering *Universities Space Research Association Visiting Scientist			
16. ABSTRACT This report summarizes various investigations into the steps commonly used to obviate gravity in isoelectric focusing. Isoelectric focusing is an equilibrium electrophoretic method in which amphoteric compounds are separated in a pH gradient according to their isoelectric values. Gravitational stabilization is required and is normally achieved by imposing a density gradient of neutrally charged carbohydrates on the pH gradient. Ficoll, a polysucrose, is commonly used in isoelectric focusing, and its influence on focused red blood cells was measured. The influence of the media pH at the cell application location was specifically determined in addition to other operational parameters, such as media properties and migration behavior of the cells.			
17. KEY WORDS		18. DISTRIBUTION STATEMENT Unclassified — Unlimited <i>Teresa Y. Miller</i>	
19. SECURITY CLASSIF. (of this r. part) Unclassified	20. SECURITY CLASSIF. (of this page) Unclassified	21. NO. OF PAGES 26	22. PRICE NTIS

ACKNOWLEDGMENTS

This work was supported by the Universities Space Research Association and the National Aeronautics and Space Administration. The authors thank Dr. Robert S. Snyder, Separation Processes Branch, Marshall Space Flight Center, for useful discussions and the provision of laboratory facilities, and Darby Mason and John R. Russell for skilled assistance.

TABLE OF CONTENTS

	Page
INTRODUCTION	1
MATERIALS AND METHODS.....	2
RESULTS AND DISCUSSION	4
CONCLUSIONS	18
REFERENCES	19

LIST OF TABLES

Table	Title	Page
1.	Electrode Solutions for Cell Isoelectric Focusing	2
2.	Sucrose-Ficoll Density Gradient Solutions	2
3.	Sucrose-Only Density Gradient Solution.....	3
4.	Effect of Ampholines on the Electrophoretic Mobility of Fresh Human RBC	7

LIST OF ILLUSTRATIONS

Figure	Title	Page
1.	Electrophoretic mobility distributions of human, turkey and chicken RBC fixed in formaldehyde. (Mobility was measured in 0.15 M NaCl at pH 7.2)	5
2.	Composition and properties of the sucrose-Ficoll density gradient for isoelectric focusing of cells designed by Boltz. [6]	6
3.	Electrophoretic mobilities of fresh human RBC in successive 5 ml fractions of a sucrose-Ficoll density gradient	7
4.	Migration distance versus time for three human RBC samples applied to a sucrose-only gradient at pH = 8.5, 7.0, and 4.5. (Ampholine pH gradient was 3.5-10)	8
5.	Migration distance versus time for three human RBC samples applied to a sucrose-Ficoll gradient at pH = 11.0, 5.4, and 2.1. (Ampholine pH gradient was 3.5-10)	9
6.	Migration distance versus time for three human RBC samples applied to a sucrose-Ficoll gradient at pH = 5.5, 2.9, and 1.9. (Ampholine pH gradient was 3-5)	10
7.	Log_{10} conductivity (N mho cm^{-1}) versus fraction number for sucrose-only and sucrose-Ficoll density gradients, focused with Ampholines pH range 3-5.....	11
8.	Distribution profile of fixed human RBC following electrofocusing in a sucrose-only pH 3-5 gradient. (8.4×10^6 RBC were applied at pH 3.9, and recovery was 64 percent).....	12
9.	Distribution profile of fixed turkey RBC following electrofocusing in a sucrose-only pH 3.5-10 gradient. (4.1×10^6 RBC were applied at pH 7.5, and recovery was 58 percent)	12
10.	Distribution profile of fixed human and turkey RBC following electrofocusing in a sucrose-only pH 3.5-10 gradient. (3.8×10^6 human RBC and 4.8×10^6 turkey RBC were applied at pH 5.8, and recoveries were 54 percent and 67 percent respectively)	13

LIST OF ILLUSTRATIONS (CONCLUDED)

Figure	Title	Page
11.	Distribution profile of fixed human and turkey RBC following electrofocusing in a sucrose-only pH 3-6 gradient. (7.0×10^6 human RBC and 3.8×10^6 turkey RBC were applied at pH 4.2, and recoveries were 71 percent and 82 percent respectively).....	13
12.	Distribution profile of fixed human and turkey RBC following electrofocusing in a sucrose-only pH 3-6 gradient. (2.7×10^6 human RBC and 3.0×10^6 turkey RBC were applied at pH 4.7, and recoveries were 54 percent and 68 percent respectively).....	14
13.	Distribution profile of fixed human and turkey RBC following electrofocusing in a sucrose-Ficoll pH 3-6 gradient. (11.0×10^6 human RBC and 4.0×10^6 turkey RBC were applied at pH 5.0, and recoveries were 73 percent and 70 percent respectively).....	14
14.	Distribution profile of fixed human and turkey RBC following electrofocusing in a sucrose-Ficoll pH 3-6 gradient. (10×10^6 human RBC and 9.6×10^6 turkey RBC were applied at pH 4.2, and recoveries were 80 percent and 87 percent respectively).....	15
15.	Distribution profile of fixed human and chicken RBC following electrofocusing in a sucrose-Ficoll pH 3-6 gradient. (5.0×10^6 human RBC and 2.9×10^6 chicken RBC were applied at pH 4.1, and recoveries were 66 percent and 77 percent respectively).....	15
16.	RBC recovery volume in fractions collected after electrofocusing versus concentration of RBC applied to sucrose-only (lower curve) and sucrose-Ficoll (upper curve) gradients	18

TECHNICAL MEMORANDUM

ISOELECTRIC FOCUSING OF RED BLOOD CELLS IN A DENSITY GRADIENT STABILIZED COLUMN

INTRODUCTION

Cell surface properties are intimately related to cell function and seem to play a fundamental role in the etiology of many disease states, including cancer. Electrophoretic studies of several cell types indicate that functional differences among normal or transformed cells are reflected in their surface properties [1,2,3]. These differences in cell electrophoretic mobility have been used as a basis for the separation and characterization of cells.

Cell electrophoresis suffers from the effects of gravity, which sediments the cells and which exacerbates the convective flows generated by Joule heat. Since cells are too large to migrate freely through the quasi-solid support media used in analytical protein electrophoresis, they must be electrophoresed in free solutions. These are stabilized against convection and cell sedimentation by being confined to thin, fast-flowing buffer films in close contact with the separation chamber walls, as in continuous flow electrophoresis [4], or by the imposition of density gradients [5,6]. Cell electrophoresis separations have also been performed on board orbiting space vehicles, and no gravitational disturbances to the process were observed [7,8,9].

Future development of zero-gravity cell electrophoresis is dependent on a thorough understanding of the terrestrial process, and to this end we have studied isoelectric focusing of cells, a method which has shown promise in lymphocyte separations [10-13].

Isoelectric focusing is an equilibrium electrophoretic method in which amphoteric compounds are separated in a pH gradient according to their isoelectric points [14,15]. Sample components migrate electrophoretically through the pH gradient until they reach a pH region corresponding to their own isoelectric pH, when their net surface charge becomes zero, and they come to rest.

Gravitational stabilization is achieved in column isoelectric focusing by imposing a density gradient of neutrally charged carbohydrates on the pH gradient. If cells are to be separated, the density gradient must be isotonic throughout its length, and this is accomplished by inverse gradients of sucrose and polysucrose (Ficoll) [6]. Ficoll is known to increase the electrophoretic mobility of red blood cells [16,17], and several studies [10-14] have reported unexpectedly high cell focusing pH.

In the present study, the possible involvement of Ficoll and cell application pH in this phenomenon were investigated by focusing red blood cells in a density gradient stabilized column. In addition, sample loading, cell dispersion, column conductivity, effect of Ampholines, and resolution of separation were examined.

MATERIALS AND METHODS

Isoelectric focusing was performed in an all-glass 110 ml LKB 8100 column, thermostatted to $4 \pm 0.5^\circ\text{C}$ using a Blue M constant temperature bath. To minimize adherence of cells to the walls of the electrofocusing column, the apparatus was immersed for 30 sec in 1 percent silicone solution (Siliclad, Clay Adams), then dried at room temperature for 24 hr. Phosphoric acid and sodium hydroxide were used as anolyte and catholyte, respectively, sucrose or sucrose-Ficoll was the density gradient material, and the pH gradients were formed with 1 percent v/v Ampholine (LKB Producter, Bromma, Sweden) with pH ranges of 3-5, 3-6, and 3.5-10. The detailed composition of electrode and gradient solutions is shown in Tables 1, 2, and 3.

TABLE 1. ELECTRODE SOLUTIONS FOR CELL ISOELECTRIC FOCUSING

Anode Solution ^a	Cathode Solution ^a
3.27 g 85 percent H_3PO_4 1.0 g Glucose Q.S. to 100 ml with distilled H_2O (40 g Sucrose if anode at bottom of column)	1.0 g NaOH Q.S. to 100 ml with distilled H_2O (40 g Sucrose if cathode at bottom of column)

a. 20 ml of each solution are used in LKB 8100 column

TABLE 2. SUCROSE-FICOLL DENSITY GRADIENT SOLUTIONS

Heavy Solution ^a	Light Solution ^a
16.7 g Ficoll (M.W.= 400,000) 7.62 g Sucrose 1.0 g Glucose 0.037 g EDTA 0.5 ml Ampholine Q.S. to 100 ml with distilled H_2O	3.33 g Ficoll 4.48 g Sucrose 1.0 g Glucose 0.037 g EDTA 0.5 ml Ampholine Q.S. to 100 ml with distilled H_2O

a. 55 ml of each solution are used in LKB 8100 column

TABLE 3. SUCROSE-ONLY DENSITY GRADIENT SOLUTION

Heavy Solution ^a	Light Solution ^a
40 g Sucrose 1.0 g Glucose 0.037 g EDTA 0.5 ml Ampholine Q.S. to 100 ml with distilled H ₂ O	1.0 g Glucose 0.037 EDTA 0.5 ml Ampholine Q.S. to 100 ml with distilled H ₂ O

a. 55 ml of each solution are used in LKB 8100 column.

Following formation of density gradients with an LKB 8121 Gradient Mixer and an LKB Varioperpex pump at a flow rate of 1.5 ml min⁻¹, the column was prefocused for 18 hr at 500 V and initial current of approximately 20 mA, using an LKB 2103 Constant Power Supply; after sample application, the voltage was raised to 1000 V, and the migration of the cells monitored visually until they reached equilibrium positions in the gradient.

Human, turkey, and chicken red blood cells were obtained by venipuncture, then washed and fixed in formaldehyde as described in Reference 18. Electrophoretic mobilities of the red cells were measured in standard saline (0.15 M NaCl adjusted to pH 7.2 ± 0.2 by addition of 0.5 M NaHCO₃) using the cylindrical-tube microelectrophoresis method described by Seaman [19].

Fixed RBC were prepared for electrofocusing by washing three times in distilled water to remove salts. The packed RBC were then resuspended in 1 ml aliquots of gradient solution, withdrawn from the prefocused pH/density gradient as described later, to a concentration not exceeding 10 × 10⁶ RBC ml⁻¹; higher cell concentrations were found to induce droplet sedimentation of the sample when applied to the column.

After focusing of cells was complete, the column contents were drained at a flow rate of 1.5 ml min⁻¹ and collected in 2.0 or 2.8 ml aliquots in an LKB 7000 fraction collector. Fraction pH was measured immediately using a PHM 64 pH meter (Radiometer, Copenhagen), and total or differential counts of unaggregated cells in each fraction were obtained using an AO Bright Line Hemocytometer.

To facilitate cell application to different regions of the preformed pH/density gradients, the electrofocusing column was modified as follows. Three lengths (3, 15, and 30 cm) of glass capillary tubing (1.0 mm ID, 1.4 mm OD) were each connected to 15 cm lengths of Tygon tubing (1.3 mm ID). The inner cooling finger of the electrofocusing column was removed, and the three Tygon tubes were inserted between the ground

glass stopper and the surface of the cooling finger, passed over the platinum electrode wire, and pushed out through the gradient inlet port. The three glass capillaries were positioned parallel to the long axis of the cooling finger, and cemented to its surface using Eastman 910 contact adhesive.

This arrangement allowed cell application, from syringes connected to each of the three capillaries, at the bottom, middle, and top of the pH/density gradient, with no disturbance to the gradient. At cell application rates less than 1 ml min^{-1} , cells were found to distribute themselves evenly in an annular band at the open end of the glass capillaries; the modification also allowed withdrawal of approximately 1 ml of column contents for measurements of pH and for suspension of cells prior to their insertion into the column.

RESULTS AND DISCUSSION

Fixed human, turkey, and chicken RBC with closely similar electrophoretic mobilities were selected as model cells in the present study. Turkey and human RBC, due to their different size and morphology, are easily discriminated in the light microscope at 400 magnifications, thereby allowing precise analysis of gradient fractions. In addition, the nucleated turkey cells provided information concerning the effects of electro-focusing on nucleated cells. Most importantly, the stability of fixed cells in non-isotonic media permitted analysis of cell electrofocusing in the presence or absence of Ficoll.

The electrophoretic mobilities of formaldehyde fixed human, turkey, and chicken RBC were measured in standard saline using microelectrophoresis. The mobility distributions of each species of RBC are shown in Figure 1. The mean mobilities of human and turkey RBC differed by $0.05 \mu\text{sec}^{-1}\text{V}^{-1} \text{ cm}$ ($-0.96 \mu\text{sec}^{-1}\text{V}^{-1}\text{cm}$ and $-1.01 \mu\text{sec}^{-1}\text{V}^{-1} \text{ cm}$, respectively). Chicken RBC showed a mean mobility of $-1.16 \mu\text{sec}^{-1}\text{V}^{-1} \text{ cm}$. The extensive overlap of the human and turkey distributions makes the separation of these cells by electrophoretic methods difficult; indeed, continuous flow electrophoresis at physiological pH did not resolve a mixture of the two populations [20]. However, the mobility difference between human and chicken RBC ($0.2 \mu\text{sec}^{-1}\text{V}^{-1} \text{ cm}$) is comparatively large, and their separation by continuous flow electrophoresis is routine.

Some properties of the sucrose-Ficoll density gradient designed by Boltz [6] are shown in Figure 2. In a vertical column, Ficoll concentration, density, and viscosity decrease in a linear fashion from the lower electrode towards the upper, while sucrose concentration increases in the same direction. The pH gradient, not shown in Figure 2, is formed by electrolysis of a heterogeneous mixture of low molecular weight polyamino, poly-carboxylic acids (Ampholines), which display a wide range of dissociation constants and high buffering capacity near their isoelectric points.

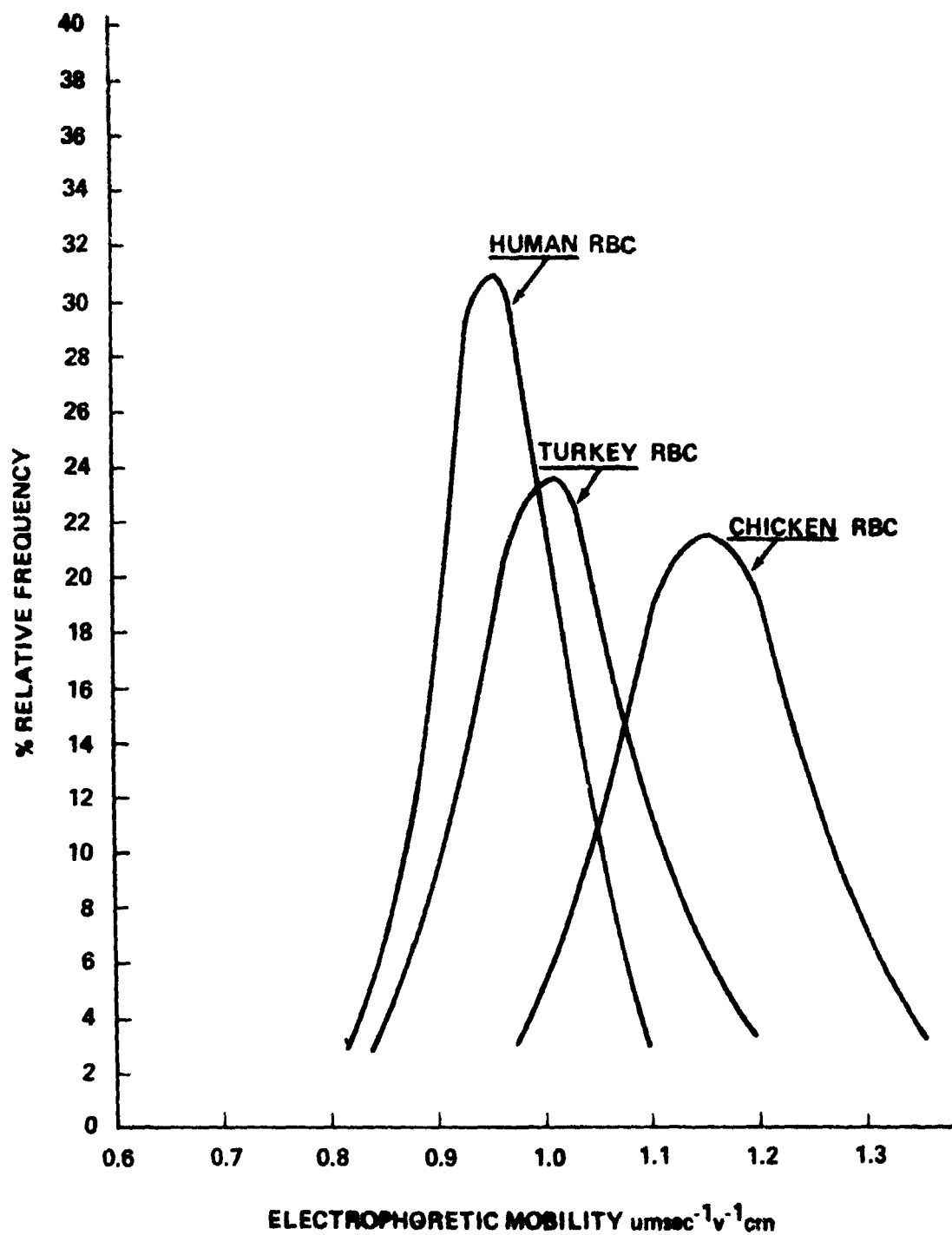


Figure 1. Electrophoretic mobility distributions of human, turkey and chicken RBC fixed in formaldehyde. (Mobility was measured in 0.15 M NaCl at pH 7.2.)

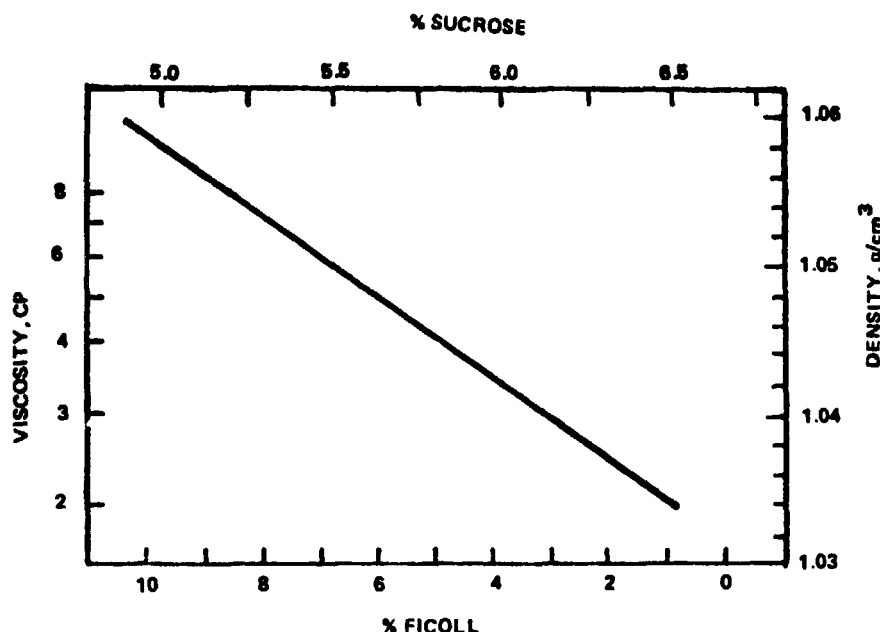


Figure 2. Composition and properties of the sucrose-Ficoll density gradient for isoelectric focusing of cells designed by Boltz [6].

Specific variations in Ampholine composition allow the formation of wide-range (pH 3.5-10) or narrow-range (pH 3-5) pH gradients. Depending on the polarity of the applied field, Ampholine gradients can either increase or decrease from the bottom of the column up. Thus cell insertion and focusing positions in the column can be varied with respect to sucrose or Ficoll concentration at constant pH, or vice versa. Field reversal in electrofocusing therefore enables discrimination between cell electrokinetic and sedimentation effects.

The electrophoretic mobilities of fresh human RBC were measured in 5 ml aliquots of standard saline to which 50 μ l of unfocused Ampholines of different pH ranges had been added. This concentration of Ampholines corresponded to the 1 percent v/v Ampholines used in electrofocusing. Despite the variation in pH of the fractions, RBC mobilities were essentially constant at the higher pH values, as shown in Table 4, suggesting that in media of high ionic strength (0.15 M), Ampholines in the higher pH range present in low concentration do not modify the electrokinetic surface structure of RBC.

The mobilities of fresh human RBC were also measured in 5 ml fractions of the sucrose-Ficoll density gradient containing no Ampholines and showing a stable pH of 7.2. Figure 3 depicts the variations of RBC mobility with fraction number, or with the sucrose/Ficoll concentrations along the length of the gradient. Decreasing Ficoll concentration was

TABLE 4. EFFECT OF AMPHOLINES ON THE ELECTROPHORETIC MOBILITY OF FRESH HUMAN RBC

Ampholines, 50 l in 5 ml 0.15 M NaCl	pH	Electrophoretic Mobility $\mu\text{msec}^{-1}\text{V}^{-1}\text{cm}$
No Ampholines	7.2	-1.08
3.5-10	7.34	-1.06
7-10	8.85	-1.08
5-7	6.05	-1.07
3.5-5	4.21	-1.04

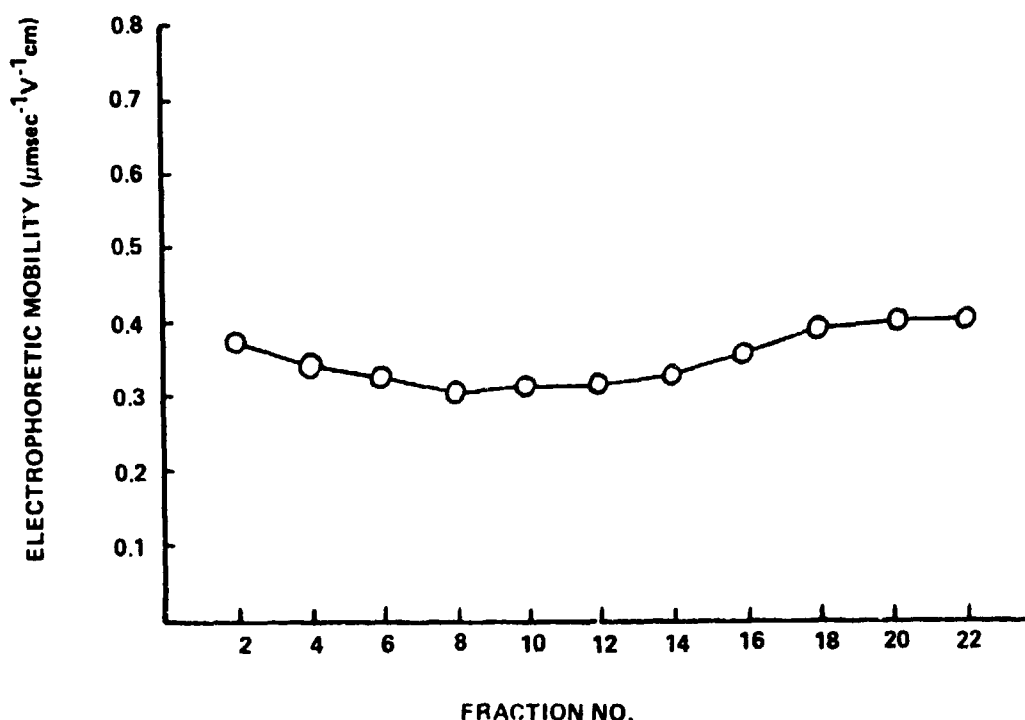


Figure 3. Electrophoretic mobilities of fresh human RBC in successive 5 ml fractions of a sucrose-Ficoll density gradient.

associated initially with declining electrophoretic mobilities; in the middle of the gradient, mobilities were approximately constant at $-0.3 \mu\text{msec}^{-1}\text{V}^{-1}\text{cm}$ and then showed a progressive increase towards the top of the gradient. This behavior could be interpreted by Ficoll adsorption to RBC's,

altering their electrophoretic mobilities, an effect which is related to polymer concentration [17]. However, the continual decline in column viscosity leads to apparent increases in cell electrophoretic mobilities, and the net effect of both phenomena appears to maintain RBC mobilities within a narrow range (-0.3 to $-0.4 \mu\text{msec}^{-1}\text{V}^{-1} \text{cm}$).

To define the contribution of Ficoll concentration to the focusing pH of cells, fixed human RBC were inserted at three different pH's (8.5, 7.0, and 4.5) into a sucrose-only stabilized column containing 1 percent v/v wide-range Ampholines (pH 3.5-10) which had been pre-focused for 18 hr, giving rise to a linear pH gradient decreasing in value towards the anode at the top of the column. After 2 hr, the three human RBC samples condensed into a single band which was recovered at pH = 3.8 after draining of column contents (Fig. 4). When a sucrose-Ficoll density gradient replaced the sucrose-only gradient, the pH gradient remaining the same (pH 3.5-10), and fixed human RBC again applied as three bands at pH 11.0, 5.4, and 2.1, only those RBC applied at the intermediate pH migrated at all, condensing after 2 hr at pH 3.7. The RBC applied at extremes of pH showed substantial aggregation and sedimentation through the gradient (Fig. 5).

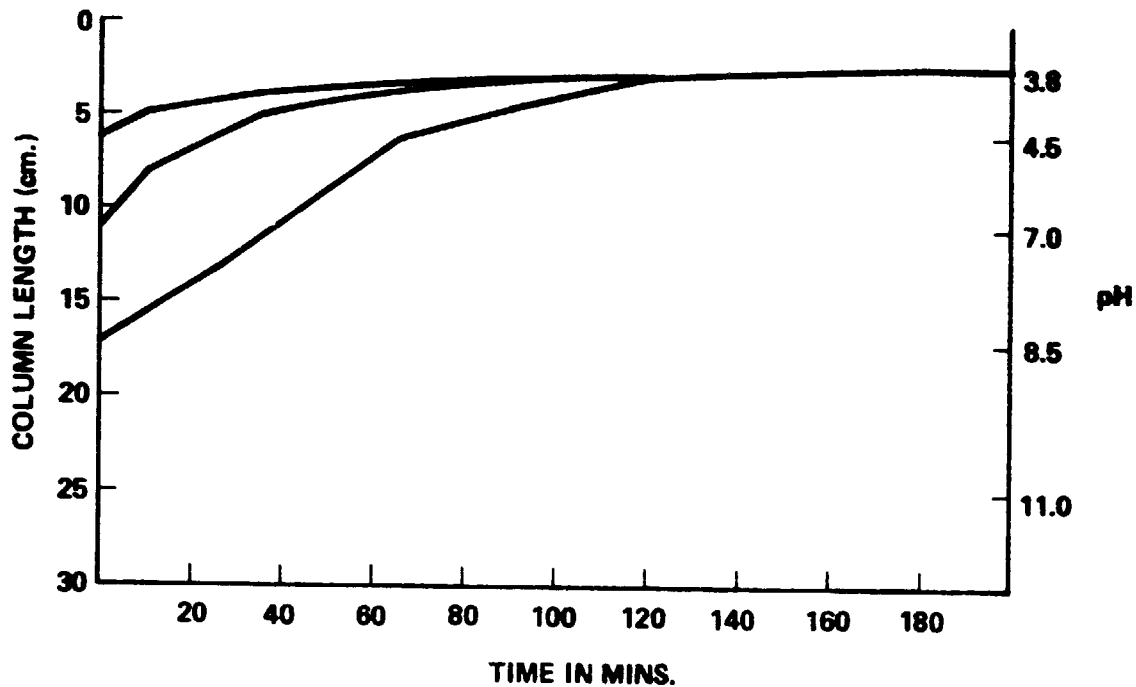


Figure 4. Migration distance versus time for three human RBC samples applied to a sucrose-only gradient at pH = 8.5, 7.0, and 4.5. (Ampholine pH gradient was 3.5-10.)

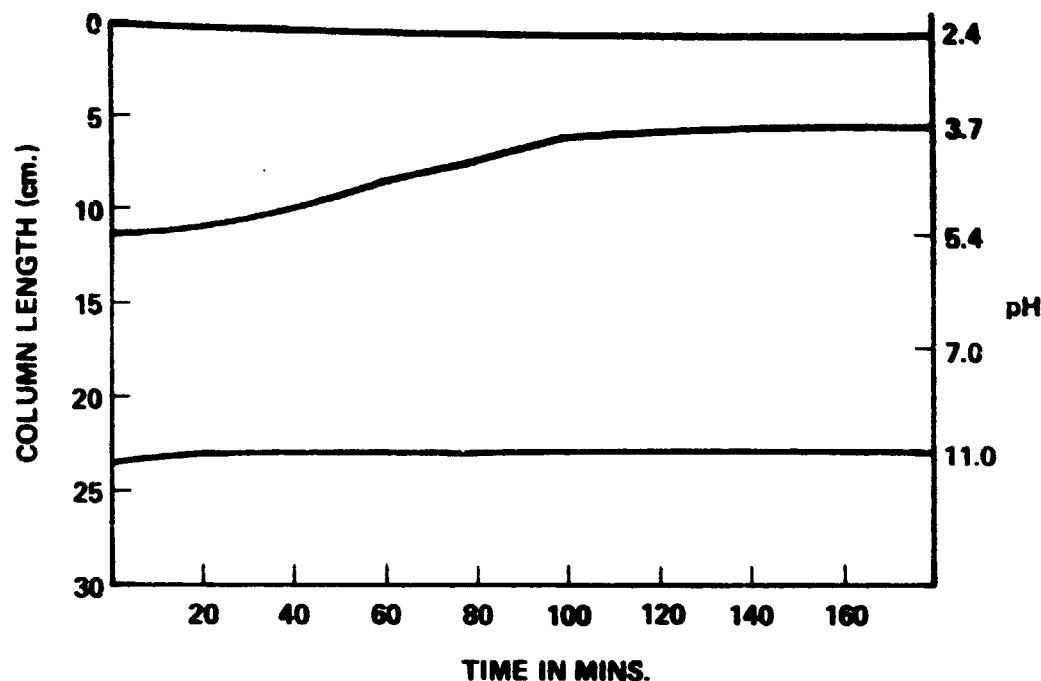


Figure 5. Migration distance versus time for three human RBC samples applied to a sucrose-Ficoll gradient at pH = 11.0, 5.4, and 2.1. (Ampholine pH gradient was 3.5-10.)

To verify the validity of using fixed cells as models for living cells in electrofocusing, this experiment was repeated with fresh human RBC. A sucrose-Ficoll gradient was prefocused with a narrow-range Ampholine pH gradient (pH 3-5). Human RBC were applied at pH = 5.5, 2.9, and 1.9, and focused for 2.5 hr. Cells inserted at pH 5.5 migrated a distance of 7 cm and were subsequently recovered at pH = 3.7, while those applied at low pH did not migrate and were recovered in an aggregated state at pH = 2.0 and 3.0 (Fig. 6).

The results of these three experiments suggested that exposure of fixed human RBC to focused Ampholines of specific differing pH's had little effect on their final focusing pH, except at extremes of pH, and that in the presence of Ficoll, the focusing pH of fresh and fixed human RBC was reduced only slightly, from pH = 3.8 to pH = 3.7. Fresh human RBC behaved similarly to fixed cells, indicating that formaldehyde fixation had no effect on their isoelectric point, at least under these electrofocusing conditions. The results also indicated that viscosity, density, and concentration of the stabilizing gradient have a minimal effect on focusing pH, since similar pH's of cell application (pH = 5.4 and 5.5) at different positions in the density gradient, i.e., at different sucrose/Ficoll concentrations, did not modify the cell focusing pH.

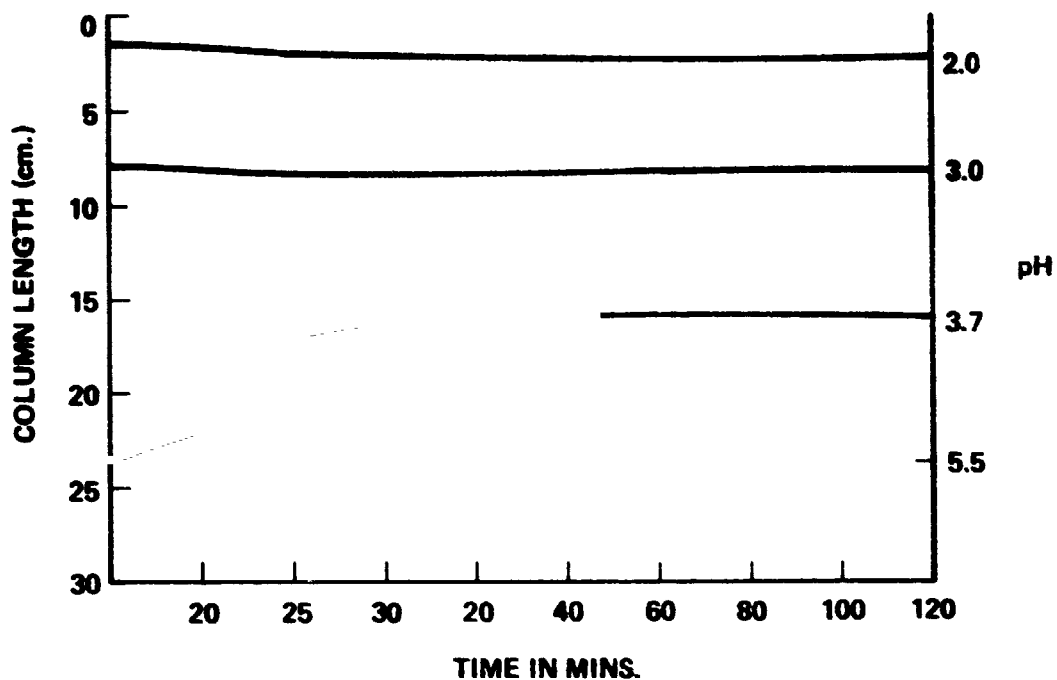


Figure 6. Migration distance versus time for three human RBC samples applied to a sucrose-Ficoll gradient at pH = 5.5, 2.9, and 1.9. (Ampholine pH gradient was 3-5.)

The extent of the charged double layer at cell surfaces is inversely proportional to the ionic strength of the suspending medium, which is therefore important in defining the cell's electrophoretic mobility and isoionic pH. To determine whether Ficoll influenced the ionic strength, and hence conductivity, of the density gradients used in electrofocusing, the conductivity of successive fractions of a prefocused narrow-range Ampholine gradient (pH 3-5) was measured in the presence and absence of Ficoll. The resulting conductivity profile, shown in Figure 7, was similar in sucrose-only and sucrose-Ficoll density gradients, and exhibited a sharp decline from the anode (acid) end of the column. The origin of this conductivity gradient is unclear. Ficoll as supplied contains small amounts of NaCl (approximately 1 percent), but this contaminant was not responsible for the observed conductivity changes, which were reproduced in sucrose-only density gradients. The conductivity gradient could be artifactual, since conductivities were measured in 2 ml fractions in the absence of an applied field, and could be due to Ampholine molecules no longer at their steady-state focusing pH. Furthermore, pH gradients formed in free solutions appear not to generate conductivity gradients. This observation is based on the results of Just et al. [20], who focused RBC in Ampholine pH gradients formed in a continuous flow electrophoresis apparatus. He found that RBC focusing pH corresponded to that calculated from the extrapolation of ionic strength/cell isoelectric

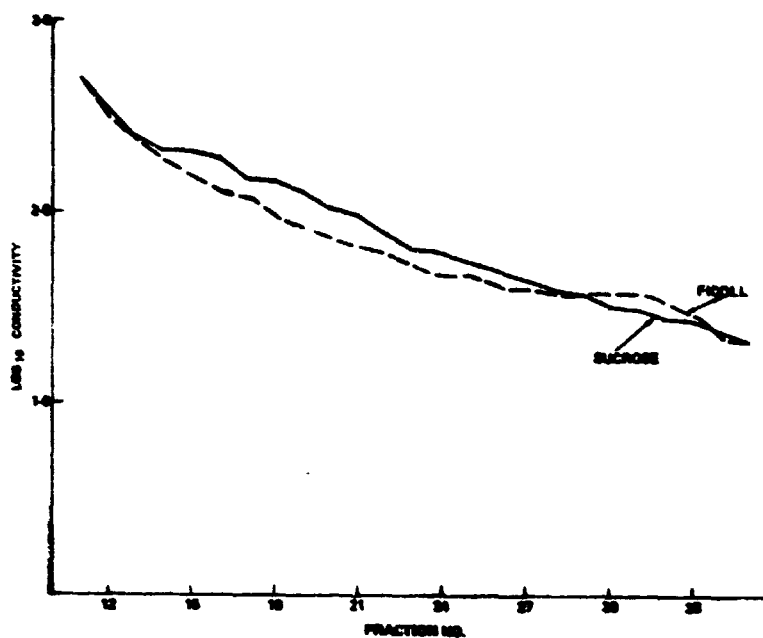


Figure 7. Log_{10} conductivity (N mho cm^{-1}) versus fraction number for sucrose-only and sucrose-Ficoll density gradients, focused with Ampholines pH range 3-5.

point data, compiled by Heard and Seaman [21], to zero ionic strength. The conductivity profiles of density gradient or gell-stabilized Ampholine pH gradients are known for their nonuniformity [22]; consequently, focusing pH in such systems does not represent isoionic pH and should be interpreted with caution.

While Ficoll seemed not to affect the focusing pH of RBC, the possibility remained that it could affect the cell dispersion in the gradient or cell stability at high concentrations. These aspects of cell electrofocusing and the specific separative capabilities of the method were explored in a further series of eight experiments. Ficoll-dependent effects were isolated by comparison of the cell distributions obtained in sucrose-only and sucrose-Ficoll density gradients. The results of these experiments are shown graphically in Figures 8 through 15; each experiment was repeated at least twice, with good reproducibility in all cases.

Human RBC (8.4×10^6) were inserted into a prefocused pH range 3-5 Ampholine gradient at pH 3.9. The gradient was stabilized with sucrose and a reversed field applied. Human RBC focused maximally at pH = 3.8 but, due in part to the shallowness of the pH gradient, were dispersed over 10 fractions, or 20 mls of gradient solution (Fig. 8). Recovery of human RBC was 64 percent, the low yield resulting from cell aggregation and sedimentation near their focusing pH (vide infra). In a steeper pH gradient (pH range 3.5-10), again with no Ficoll, turkey

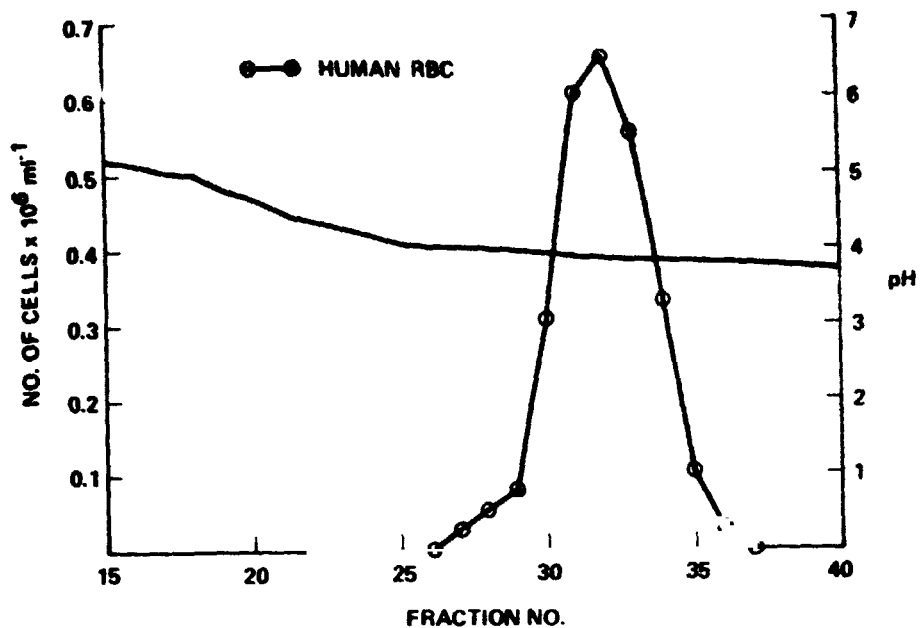


Figure 8. Distribution profile of fixed human RBC following electrofocusing in a sucrose-only pH 3-5 gradient. (8.4×10^6 RBC were applied at pH 3.9, and recovery was 64 percent.)

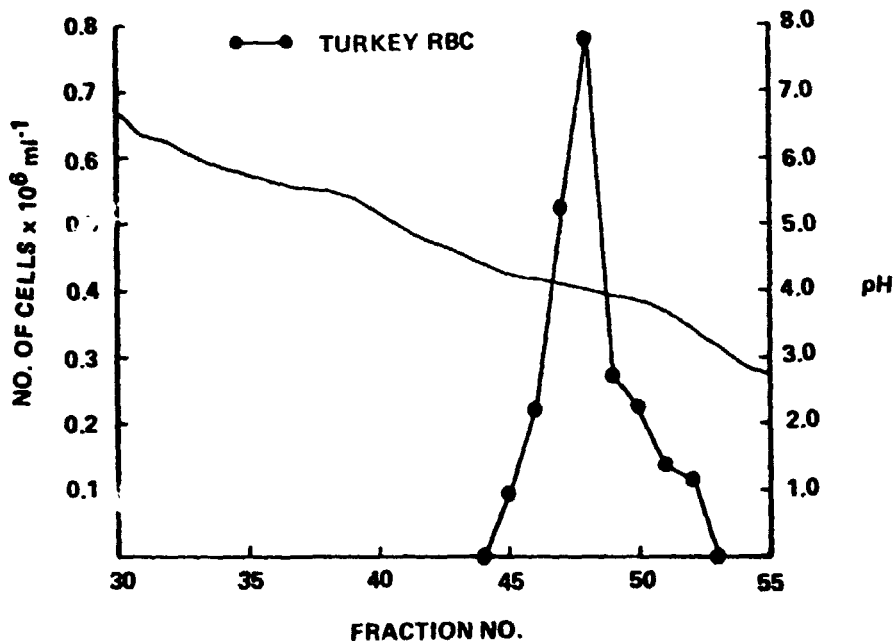


Figure 9. Distribution profile of fixed turkey RBC following electrofocusing in a sucrose-only pH 3.5-10 gradient. (4.1×10^6 RBC were applied at pH 7.5, and recovery was 58 percent.)

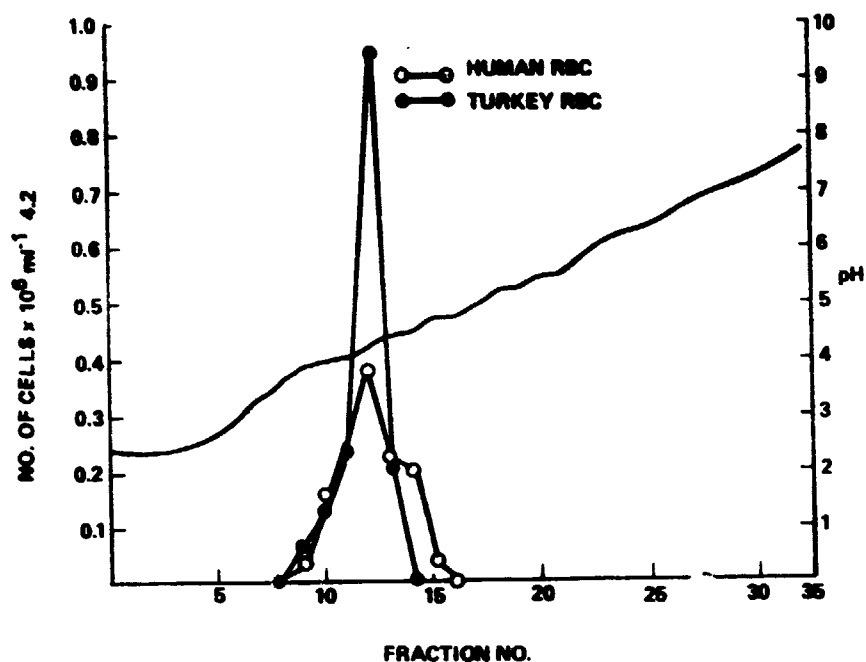


Figure 10. Distribution profile of fixed human and turkey RBC following electrofocusing in a sucrose-only pH 3.5-10 gradient. (3.8×10^6 human RBC and 4.8×10^6 turkey RBC were applied at pH 5.8, and recoveries were 54 percent and 67 percent respectively.)

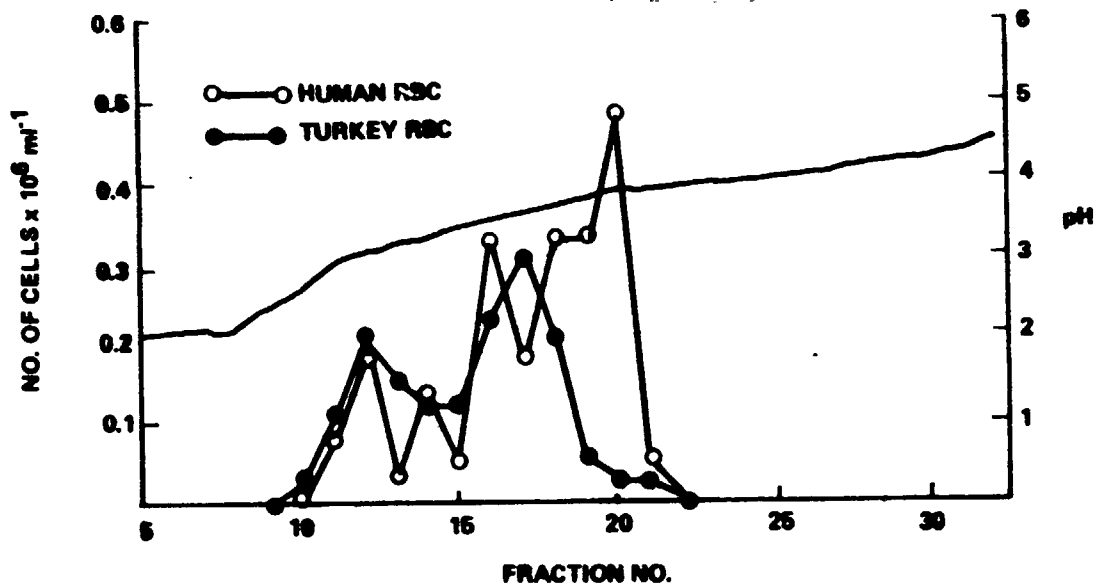


Figure 11. Distribution profile of fixed human and turkey RBC following electrofocusing in a sucrose-only pH 3-6 gradient. (7.0×10^6 human RBC and 3.8×10^6 turkey RBC were applied at pH 4.2, and recoveries were 71 percent and 82 percent respectively.)

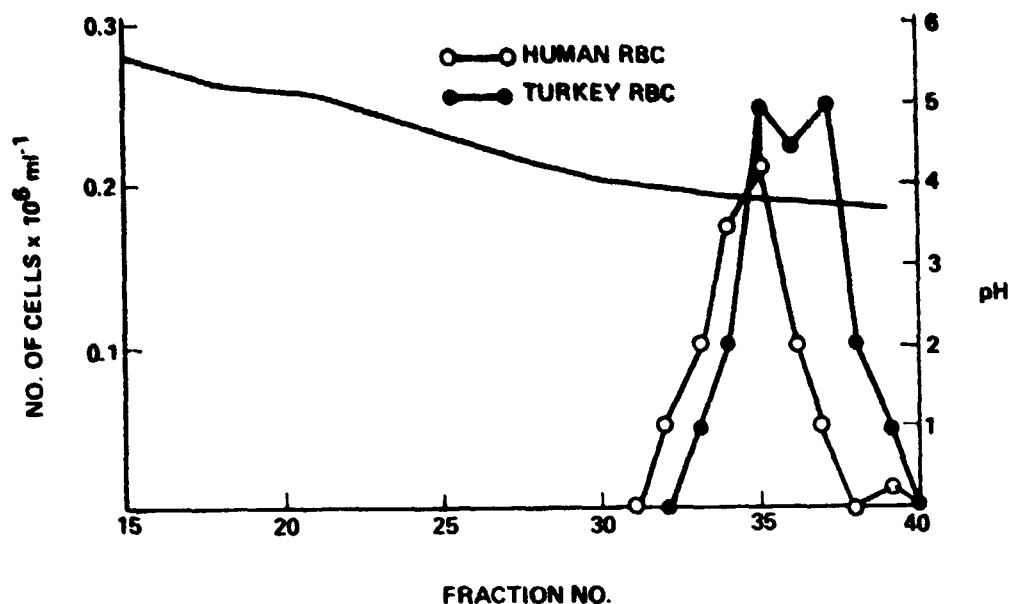


Figure 12. Distribution profile of fixed human and turkey RBC following electrofocusing in a sucrose-only pH 3-6 gradient. (2.7×10^6 human RBC and 3.0×10^6 turkey RBC were applied at pH 4.7, and recoveries were 54 percent and 68 percent respectively.)

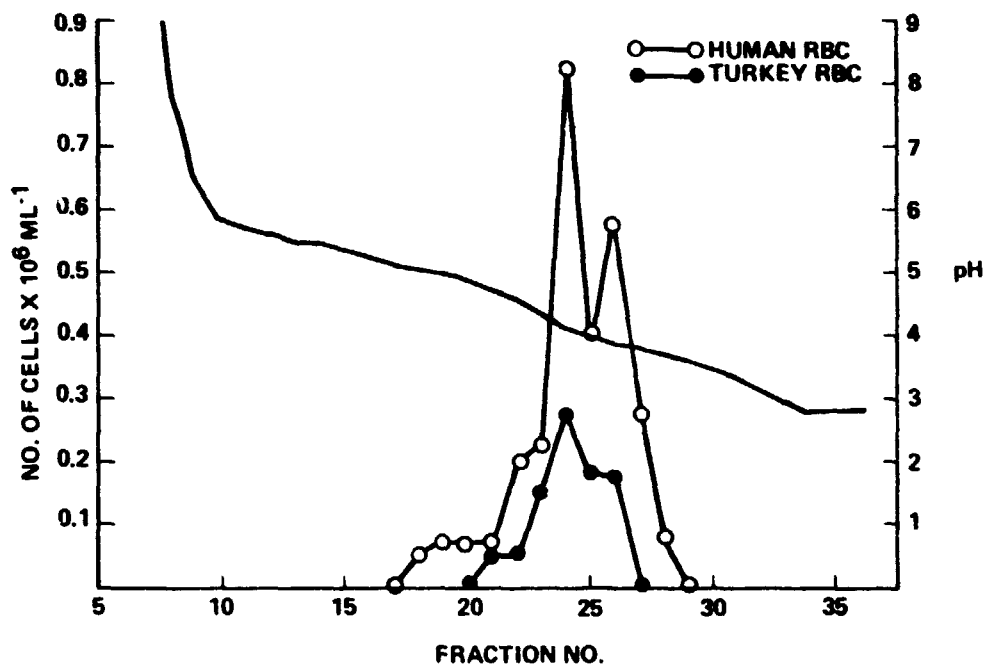


Figure 13. Distribution profile of fixed human and turkey RBC following electrofocusing in a sucrose-Ficoll pH 3-6 gradient. (11.0×10^6 human RBC and 4.0×10^6 turkey RBC were applied at pH 5.0, and recoveries were 73 percent and 70 percent respectively.)

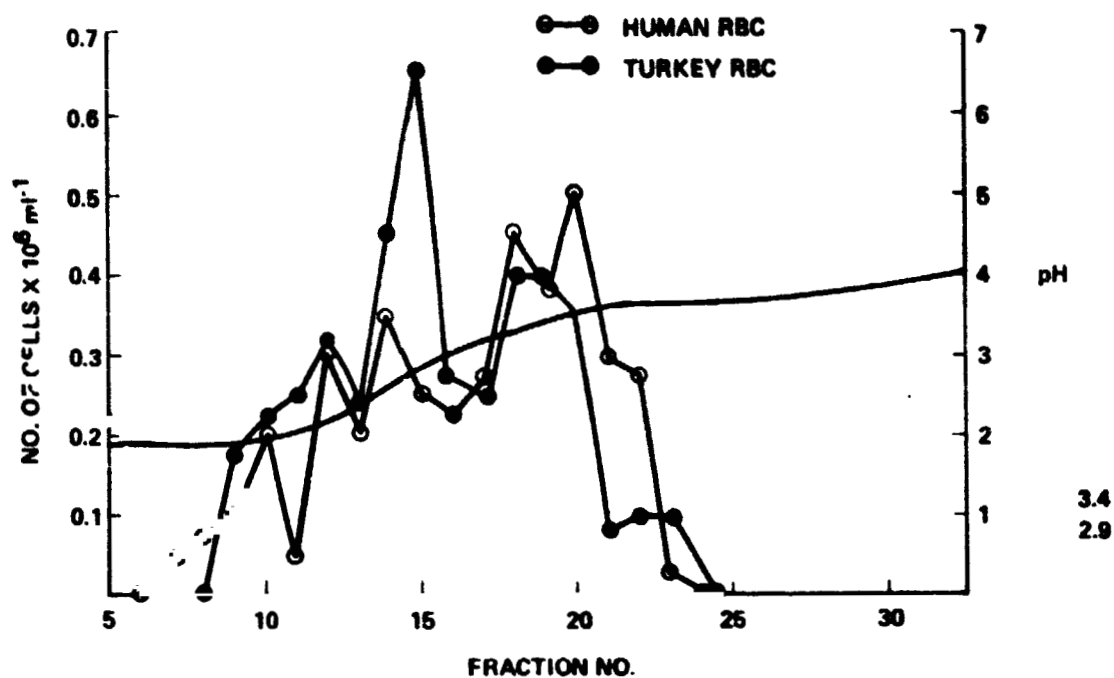


Figure 14. Distribution profile of fixed human and turkey RBC following electrofocusing in a sucrose-Ficoll pH 3-6 gradient. (10×10^6 human RBC and 9.6×10^6 turkey RBC were applied at pH 4.2, and recoveries were 80 percent and 87 percent respectively.)

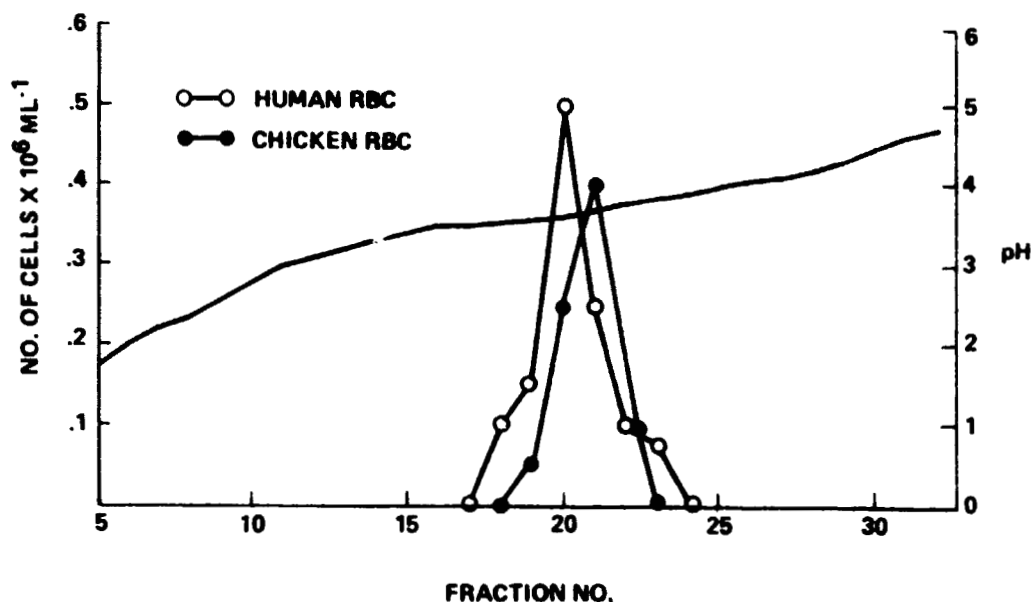


Figure 15. Distribution profile of fixed human and chicken RBC following electrofocusing in a sucrose-Ficoll pH 3-6 gradient. (5.0×10^6 human RBC and 2.9×10^6 chicken RBC were applied at pH 4.1, and recoveries were 66 percent and 77 percent respectively.)

RBC applied at pH = 7.5 condensed to a single band at pH = 4.0, with a dispersion covering 8 fractions (Fig. 9). Cell recovery was 58 percent.

When human and turkey RBC were applied at a concentration of 8.5×10^6 total cells per ml at pH 5.8 to a prefocused steep pH gradient (pH range 3.5-10) in a sucrose-only density gradient, they migrated together and came to equilibrium within 2 hr. Analysis of gradient fractions showed a focusing pH for both cell types of pH = 4.1, and no separation (Fig. 10). Human and turkey RBC recoveries were 54 percent and 67 percent, respectively.

Since shallow pH gradients are in principle capable of greater resolution, the experiment was repeated in a pH range 3-6 prefocused Ampholine gradient, with an RBC application pH of 4.2. No Ficoll was used in the density gradient. Analysis of the RBC distribution recovered after focusing showed differentiation of human and turkey RBC into two sub-populations (Fig. 11). However, the RBC distributions as a whole were superimposed, with the turkey RBC being recovered only slightly closer to the anode than the human RBC. The appearance of human and turkey RBC sub-populations in this experiment was tentatively attributed to high sucrose concentration at the focusing pH, since this resolution was not observed with human RBC in an otherwise similar pH/density gradient (Fig. 8). Owing to the reversed field in that experiment, RBC focused at pH = 3.9 were recovered in fractions of relatively low sucrose concentration. To assess further the role of sucrose in sub-population discrimination, another sample of human and turkey RBC (5.7×10^6 total RBC per ml) was applied to a sucrose-only stabilized column at pH = 4.7 (reversed field, low sucrose concentration) in a pH range 3-6 prefocused gradient (Fig. 12). Slight resolution of human and turkey RBC was evident in the collected fractions, but human and turkey sub-populations were not well resolved. Despite the field reversal, turkey RBC were again recovered closer to the anode than human RBC, confirming the electrokinetic basis of their partial resolution, rather than density sedimentation effects. Human and turkey RBC recoveries were 54 percent and 68 percent, respectively, and the cells were distributed over 8 fractions, or 16 mls of gradient solution.

When a sucrose-Ficoll density gradient was substituted for the sucrose-only gradient under the same experimental conditions (Ampholine pH range 3-6, sample insertion at pH = 5.0), human and turkey RBC both showed a bimodal distribution, although total cell distributions were again superimposed, i.e., human and turkey RBC were not separated from one another (Fig. 13).

Repetition of the experiment with field reversal, finer fractionation of the gradient (2 ml rather than 2.8 ml fractions were collected), and RBC insertion close to their focusing pH again revealed the presence of human and turkey sub-populations, although neither cell species was separated from the other (Fig. 14).

Since analytical microelectrophoresis of human and turkey RBC at pH = 7.2 shows them to possess unimodal Gaussian distributions (Fig. 1), resolution of both RBC's into bimodal distributions in density gradient electrofocusing could be artifactual. The apparent dependence of the phenomenon on differences in sucrose concentration at the focusing pH of the cells (pH = 3.8) suggests a density sedimentation effect. Continuous flow electrofocusing of human RBC in pH 3-6 Ampholine gradients gave unimodal RBC distributions [20].

In the experiments thus far described, separation of human and turkey RBC differing in mobility by $0.05 \mu\text{sec}^{-1}\text{v}^{-1}\text{cm}$ was not achieved. To define more closely the resolving power of cell electrofocusing, fixed human and chicken RBC showing a larger mobility difference of $0.2 \mu\text{sec}^{-1}\text{v}^{-1}\text{cm}$ were applied at pH = 4.1 to a sucrose-Ficoll density gradient, prefocused for 18 hr with pH range 3-5 Ampholines. After 3 hr, two bands of cells were observed at equilibrium, 2.0 mm apart, close to the original point of insertion. The recovered cell distribution is shown in Figure 15. Human RBC were found in highest concentration in fraction 20 (36 percent of human RBC applied) at a pH of 3.6, while chicken cells focused maximally at pH = 3.7. Both cell species were recovered in their original proportions in 5 fractions, and pure human RBC were recovered in low concentration the sixth fraction. Although visual resolution in the column was good, flow disturbances to the gradient, incurred during draining of the column, resulted in poor resolution of the cells after collection.

Finally, the effects of density gradient composition on cell dispersion during electrofocusing were assessed by correlating RBC concentrations applied to the column with the total volume of cell-containing fractions recovered after focusing. The results are shown in Figure 16. In sucrose-only gradients, cell dispersion was approximately constant despite variations in RBC concentrations applied to the gradients. In these gradients, approximately 40 percent of the cells applied were lost through aggregation and sedimentation. In sucrose-Ficoll gradients, however, cell dispersion after focusing was proportional to the applied cell concentrations, and only 20 percent of the cells were lost by aggregation. Since most biological materials exhibit minimum solubility at their isoelectric point, which in the case of cells is expressed as aggregation and subsequent precipitation from suspension, these results indicated that Ficoll conferred some degree of stabilization on suspended cells near their isoelectric point. This finding is consistent with studies of erythrocyte aggregation in the presence of dextran which indicated that intercellular electrostatic repulsion was enhanced by the presence of adsorbed natural polymer [17]. However, the increased cellular dispersion observed in sucrose-Ficoll gradients, particularly at applied cell concentrations in excess of $10 \times 10^6 \text{ ml}^{-1}$, tends to compromise resolution, since closely adjacent sample bands will overlap to a greater extent.

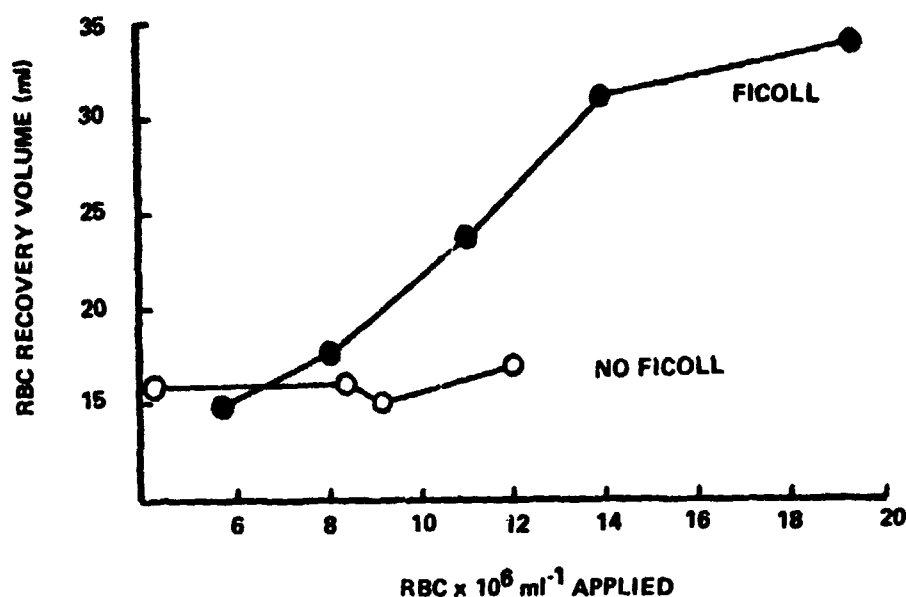


Figure 16. RBC recovery volume in fractions collected after electrofocusing versus concentration of RBC applied to sucrose-only (lower curve) and sucrose-Ficoll (upper curve) gradients.

CONCLUSIONS

Preparative scale cell separation using static column isoelectric focusing with Ampholine pH gradients has proven difficult for two reasons. Firstly, Ampholine interactions with the surface of RBC may introduce homogeneity into an otherwise heterogeneous cell population, effectively limiting the resolution of the focusing system. Secondly, the upper limit of cell concentration that can be applied to the gradient is limited by droplet sedimentation and dispersion to 10^{-7} ml⁻¹. Although Ficoll adsorbs to cells, it has a minimal effect on their focusing pH, and also stabilizes cells against aggregation. The occurrence of a substantial conductivity gradient in electrofocusing and the apparent resolution of RBC into sub-populations remain unclarified, although both phenomena are absent in free solution electrofocusing.

REFERENCES

1. Ambrose, E. J.: *Cell Electrophoresis*. Little, Brown and Co., Boston, 1965.
2. Wallach, D. F. H. and Perez-Esandi, M. V.: *Biochim. Biophys. Acta.* 93:363, 1964.
3. Ruhenstroth-Bauer, G., Fuhrmann, G. F.; Grunzer, E.; Kuebler, W.; and Rueff, F.: *Bioscience.* 16:335, 1966.
4. Hannig, K.: *Methods in Microbiology*. Vol. 5B (J. R. Norris and D. W. Ribbons, eds.) Academic Press, New York, 1971.
5. Mel, H. C.: *J. Theoret. Biol.* 6: 307, 1964.
6. Boltz, R. C.; Todd, P.; Steibel, M. J.; and Louie, M. K.: *Prep. Biochem.* 3(4): 383-401, 1973.
7. McKannan, E. C.; Krupnik, A. C.; Griffin, R. N.; and McCreight, L. R.: *NASA TMX-64611*, 1971).
8. Snyder, R. S.; Bier, M.; Griffin, R. N.; Johnston, A. J.; Leidheiser, H.; Micale, F. J.; Ross, S.; and Van Oss, C. J.: *Sep. Purif. Methods.* 2: 259-282, 1973.
9. Allen, R. E.; Barlow, G. H.; Bier, M.; Bigazzi, P. E.; Knox, R. J.; Micale, F. J.; Seaman, G. V. F., Vanderhoff, J. W.; Van Oss, C. J.; Patterson, W. J.; Scott, F. E.; Rhodes, P. H.; Nerren, B. H.; and Harwell, R. J.: *NASA TMX-58173*, 1976.
10. Sherbert, G. V.; Hakshmi, M. S.; and Rao, K. V.: *Exp. Cell. Res.* 70: 113-123, 1972.
11. Leise, E. M. and Le Sane, F.: *Prep. Biochem.* 4: 395, 1974.
12. Hirsch, R. L.; Gray, I.; and Bellanti, J. A.: *J. Immunol. Methods.* 18: 95-104, 1977.
13. Boltz, R. C.; Todd, P.; Hammerstedt, R. H.; Hymer, W. C.; Thomson, C. J.; and Docherty, J.: *Cell Separation Methods*. (H. Bloemendal, ed.) Elsevier/North Holland Biomedical Press, p. 145, 1977.
14. Svenssen, H.: *Acta Chim. Scand.* 15: 325-341, 1961.
15. Vesterberg, O. and Svenssen, H.: *Acta. Chim. Scand.* 20: 829-834, 1966.

REFERENCES (CONCLUDED)

16. Pollack, W.; Hager, H. J.; Reckel, R.; Toren, D. A.; and Singher, H. O.: *Transfusion*. 5: 158, 1965.
17. Brooks, D. E. and Seaman, G. V. F.: *J. Colloid Interface Sci.* 43: 670, 1973.
18. Heard, D. H. and Seaman, G. V. F.: *Biochim. Biophys. Acta.* 53: 366-374, 1961.
19. Seaman, G. V. F.: *Cell Electrophoresis*. (E. J. Ambrose, ed.) p. 4, J. & a. Churchill, London, 1965.
20. Just, W. W.; Leon, J. O., V; and Werner, G.: *Anal. Biochem.* 67: 590-601, 1975.
21. Heard, D. H. and Seaman, G. V. F.: *J. Gen. Physiol.* 4: 15, 1960.
22. Righetti, P. G.; Balzarini, L.; Gianazza, E.; and Brenna, O.: *J. Chromatog.* 134: 279, 1977.

APPROVAL

ISOELECTRIC FOCUSING OF RED BLOOD CELLS IN A DENSITY GRADIENT STABILIZED COLUMN

By Adam J. K. Smolka and Teresa Y. Miller

The information in this report has been reviewed for technical content. Review of any information concerning Department of Defense or nuclear energy activities or programs has been made by the MSFC Security Classification Officer. This report, in its entirety, has been determined to be unclassified.



ROBERT S. SNYDER
Chief, Separation Processes Branch



ROBERT J. NAUMANN
Chief, Space Processing Division



CHARLES A. LUNDQUIST
Director, Space Sciences Laboratory